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Salt tolerance of halophytes, research questions reviewed in the perspective of saline agriculture[☆]

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ABSTRACT

Halophytes of the lower coastal salt marsh show increased salt tolerance, and under high salinity they grow faster than upper marsh species. We could not show reduced growth rate of halophytes compared with glycophytes when grown under non-saline conditions. This indicates limited energy costs associated with high-salt tolerance in plants of genera such as *Salicornia*, providing a good perspective of saline agriculture cultivating *Salicornia* as a vegetable crop.

We show that halophytes do not occur on non-saline or inland sites because of a reduced growth rate at low soil salinity, but probably due to other ecological traits of glycophytic upper marsh species. These traits provide competitive advantage over lower salt marsh halophytes, such as earlier germination and increased growing season length.

Some halophytic Amaranthaceae (Salicornioideae, Chenopodioideae and Suaedoideae) are not just highly salt tolerant, their growth rate is stimulated at a salinity range of 150–300 mM NaCl. Alternatively this may be described as depressed growth at low salinity.

Selective pressure for such high-salt tolerance and salt stimulated growth likely occurred with prevailing arid climate and saline soil conditions. Under such conditions highly-salt tolerant succulent Salicornioideae, Chenopodioideae and Suaedoideae may have evolved about 65 Mya. In the context of evolution and diversification of land plants this origin of highly-salt tolerant succulent plants is relatively recent.

Such high-salt tolerance might be characterized as constitutive in comparison with inducible (lower) salt tolerance of other dicotyledonae and monocotyledonae (Poaceae) species. Levels of salt tolerance of the latter type span a large range of low, intermediate to high-salt tolerance, but do not include salt stimulated growth. Salt tolerant traits of the latter inducible type appear to have evolved repeatedly and independently.

Early highly-salt tolerant succulent Salicornioideae, Chenopodioideae and Suaedoideae were perennial and frost sensitive and occurred in warm temperate and Mediterranean regions. A shift from the perennial *Sarcocornia* to an annual life form has been phylogenetically dated circa 9.4–4.2 Mya and enabled evolution of annual hygrohalophytes in more northern coastal locations up to boreal and subarctic coastal sites avoiding damage of winter frost. Diversification of such hygrohalophytes was facilitated by polyploidization (e.g. occurrence of tetraploid and diploid *Salicornia* species), and a high degree of inbreeding allowing sympatric occurrence of *Salicornia* species in coastal salt marshes.

High-level salt tolerance is probably a very complex polygenic trait. It is unlikely that glycophytes would accommodate the appropriate allelic variants at all the loci involved in halophyte salt tolerance. This might explain why attempts to improve crop salt tolerance through conventional breeding and selection have been unsuccessful to date.

Genetic engineering provides a viable alternative, but the choice for the appropriate transgenes is hampered by a fundamental lack of knowledge of the mechanisms of salt tolerance in halophytes. The chances to identify the determinant genes through QTL analyses, or comparisons among near isogenic lines (NILS) are limited. Salt-tolerance is usually a species-wide trait in halophytes, and intra-specific divergence in salt tolerance in facultative halophytes seems to be often associated with chromosomal incompatibility.

[☆] In memory of Professor Yoav Waisel (1931–2010) who greatly contributed to the science of halophytes and development of saline agriculture.

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A variety of candidate salt tolerance genes been identified in *Arabidopsis thaliana*, among which genes encoding Na⁺ and K⁺ transporters, and genes involved in the general stress or anti-oxidant response, or in compatible solute metabolism. Many of these genes have been over-expressed in different glycophytic hosts, which usually appeared to alleviate, to some degree, the response to high salinity levels. However, with few exceptions, there are no indications that the same genes would be responsible for the superior salt tolerance in (eu)halophytes. Comparisons of gene expression and gene promoter activity patterns between halophytes and glycophytes are, with few exceptions, virtually lacking, which is a major omission in current day salt tolerance research.

Full-genome transcriptomic comparisons between halophytes and related glycophytes through deep sequencing seem to be the most promising strategy to identify candidate genetic determinants of the difference in salt tolerance between halophytes and glycophytes.

The most reliable validation of any candidate gene is through silencing the gene in the halophytic genetic background, preferably down to the level at which it is expressed in the glycophyte reference species. This requires genetically accessible halophyte models, which are not available to date, with the exception of *Thellungiella halophila*. However, more models are required, particularly because *T. halophila* is not a typical halophyte. Eventually, the pyramiding of validated salt tolerance genes under suitable promoters may be expected to be a viable strategy for crop salt tolerance improvement.

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1. Introduction and aim of this paper

We briefly review selected developments of halophyte and salt tolerance research. Of course the selection of research topic remains arbitrary, but we consider at least some of them of general importance. In particular the following research questions will be addressed.

(a) Is there a trade-off between high-salt tolerance and (reduced) relative growth rate? (b) Do we understand salt stimulated growth in highly salt tolerant halophytes? (c) Do we understand why salt stimulated growth (and succulence) does hardly, if at all, occur in monocotyledonous (grasses, Poaceae) halophytes? Or otherwise: just occurs in dicotyledonous plant groups? (d) How and when did salt tolerance in terrestrial higher plants evolve, with focus on highly salt tolerant Chenopodiaceae, Salicornioideae and Suaedoideae, (e) Why did attempts to improve the salt tolerance of salt-sensitive crops through conventional breeding during the last decades fail? (f) Can we suggest a viable strategy for future engineering of improved salt tolerance? (g) Is there a new perspective for a successful saline agriculture?

We do not intend to review fully the literature with regard to the above research topics, but attempt to critically discuss and analyze these topics and, if possible, bring forward new research ideas.

Although this analysis primarily starts from ecophysiological, molecular genetic phylogenetic and viewpoints, our context and final aim is to support the cultivation of crops irrigated with brackish and saline water, i.e. saline agriculture.

Both the critical analysis of published literature on halophytes and salt tolerance and the development of new research ideas contain speculative elements. We sincerely hope that our suggestions will form part of new experimental and field research on halophytes and saline crops.

2. Is there a trade-off between high-salt tolerance and (reduced) growth rate?

In salt marshes coastal plants do not just survive at certain salinity levels. They also grow and reproduce vegetatively and sexually and generally there is distinct species zonation from lower to upper parts of the salt marshes (Cooper, 1982; Schat, 1982; Rozema et al., 1985). Ranked along an elevational gradient, plant species from the lower marsh have often been found more salt and flood tolerant than plants from the middle and upper marsh (Flowers et al., 1977, 1986a,b; Rozema, 1978; Cooper, 1982; Rozema et al., 1985; van Diggelen, 1988; Colmer and Flowers, 2008). But why do most lower salt marsh species (halophytes) not occur at the upper marsh or on less saline and non-saline inland sites? Generally it is assumed that

they are outcompeted by faster growing non-halophytes, i.e. glycophytes. The latter could imply that the (maximum) relative growth rate (RGR) of lower marsh halophytic plants at low salinity would be less than that of (glycophytic) upper marsh and non-coastal plants. However RGR values (0 mM NaCl, hydroponic culture) of 10 euhalophytes and 10 'miohalophytes' (i.e. with a lower salt tolerance and no salt induced growth), reported by Glenn and O'leary (1984) did not significantly differ (one way ANOVA, $p = 0.381$). Similarly RGR values of lower marsh species (higher salt tolerance), i.e. *Salicornia* spp., *Spartina anglica*, *Aster tripolium*, *Atriplex portulacoides*, *Limonium vulgare*, *Triglochin maritima*, *Spergularia maritima* and upper marsh species (lower salt tolerance), i.e. *Elymus pycnanthus*, *Elymus repens*, *Festuca rubra*, *Agrostis stolonifera*, *Atriplex littoralis*, *Plantago lanceolata*, *Chenopodium album*) salt marsh plants obtained by van Diggelen (1988) did not differ (one way ANOVA, $p = 0.330$, Table 1). Table 1 also implies that the maximum RGR of a lower salt marsh species, such as *Salicornia* spp., is not necessarily lower than that of glycophytic (non halophytic) plants species. This conclusion was also reached by Flowers and Colmer (2008). This finding offers a good perspective for the productivity and yield of *Salicornia* cultivation in saline agriculture (see Section 8.1).

Unfortunately, the literature provides only few reliable and useful RGR data in relation to NaCl levels in hydroponic solutions. Often salt tolerance is not calculated as the ratio between RGR values at increased and low salinity, but as, for example, shoot biomass, shoot length or yield at the final harvest. As the difference in shoot biomass, shoot length or yield at the final harvest between increased and low salinity exposure, will generally vary with duration of the experiment, the calculated salt tolerance will vary with time. Also relative growth rates based on parts of the whole plant should not be used for salt tolerance calculations. Based on the above, the majority of published salt tolerance values suffer from this bias. As a consequence, the extent of salt stimulated growth in the yield-salinity response curves presented in Fig. 1 is affected by this error e.g. dry mass at 20 dS m⁻¹ representing 170% as compared to dry mass at 0 dS m⁻¹, as well as the slopes of the rear end of these curves. With a longer exposure to increased salinity the difference between yield obtained with salt treatment and control will increase. As a result, salt tolerance assessed as the ratio (yield at increased salinity)/(yield at low salinity) will decrease and the slope of reducing yield versus increasing salinity will become steeper. Also the extent of growth stimulation will exceed 170% (cf. Maas and Hoffman, 1977).

Since, as a rule, lower marsh plants have been shown to be more salt tolerant than upper marsh plants and inland plants (van Diggelen, 1988), the apparent lack of considerable and consistent differences in the relative growth rates among these plant types

Table 1

Comparison of relative growth rate of (A) miohalophytes (lower salt tolerance) and euhalophytes (higher salt tolerance) and (B) of upper marsh species (lower salt tolerance) and lower marsh species (higher salt tolerance).

	Plant group	Mean RGR 0 mM NaCl and SEM	ANOVA	Reference
A	Miohalophytes (glycophytes) (n = 10)	0.425(0.045) mg/g/wk ⁻¹	p = 0.381	Glenn and O'leary (1984)
	Euhalophytes (n = 10)	0.372(0.038) mg/g/wk ⁻¹		
B	Upper marsh species (n = 7)	0.070 (0.005) mg/g/day ⁻¹	p = 0.330	van Diggelen (1988)
	Lower marsh species (n = 9)	0.061 (0.007) mg/g/day ⁻¹		

indicates that the energy cost of high-level salt tolerance is not particularly high. As a consequence, in general, coastal halophyte communities exhibit relatively high annual biomass productivities (Flowers and Colmer, 2008), in contrast with plant communities from dry, cold or infertile environments (Grime, 1979).

The relative growth rate (at 0 mM NaCl) as well as the salt tolerance (measured as the ratio RGR 400 mM NaCl/RGR 0 mM NaCl) of fodder beet, originating by domestication from seabed (*Beta vulgaris* ssp. *maritima*) does not differ from its salt marsh ancestor (Rozema et al., 1993; Niazi, 2007). This indicates that domestication, in this case selection for a reduced number of upright leaves with an increased leaf area and strong beet development, has not negatively affected salt tolerance. Again, this finding represents a favorable perspective for saline agriculture in need of fast growing salt tolerant crops.

2.1. Salt tolerance and the spatial distribution of halophytes and glycophytes

It does not appear that a reduced RGR is causing halophytes to be outcompeted by glycophytes in non-saline environments. What other factors might be involved? We suggest that phenological, ecological and genetic traits, such as earlier germination, an advanced start of the reproductive phenology and vegetative growth and delayed senescence (i.e. a longer growing season experienced by upper marsh plants), may provide a competitive advantage for upper marsh plants (Rozema et al., 1987a,b; Scholten et al., 1987; Rozema et al., 1988). The upper marsh is less frequently seawater flooded and the duration of the inundation period is less than that of the lower marsh. In addition, upper marsh soil is less moist than lower marsh soil and warms up faster enabling an earlier start and faster vegetative growth.

Scholten and Rozema (1990) demonstrated that three weeks earlier start of the growing season at a location along the coast in the south of the Netherlands compared to a 300 km more northern seaside location could account for the dominance of *S. anglica* in the lower parts of salt marshes in the southern Netherlands. Also other factors in the winter and summer period (duration and alternation of dry and wet (flooding) periods, frost and grazing) may affect the distribution limits of salt marsh species along elevational and geographical gradients.

3. Unique high-salt tolerance and salt stimulated growth of Salicornioideae, Chenopodioideae and Suaedoideae?

3.1. Obligate halophytes, salt stimulated growth, depressed growth at low salinity, or an artifact?

Salicornioideae and some other genera within the Chenopodioideae and Suaedoideae appear to be the only angiosperms with species showing to be 'obligate halophytes' (Barbour, 1970). Flowers et al. (1977) defined this as an obligate requirement for high ion concentrations for optimal growth. That is: these halophytes do not just tolerate high salinity levels (i.e. 400–600 mM NaCl, representing seawater level salinity) in hydroponic culture. They also require considerable salinity levels (i.e. 200–300 mM NaCl which is 35–55‰ seawater salinity see Katschnig et al., 2012) to attain optimal growth. Katschnig et al. (2012) found the RGR of *Salicornia dolichostachya* at 300 mM NaCl to be 123% of that at 50 mM NaCl. This holds for dry mass and for ash free dry mass meaning that the growth stimulation at 300 mM NaCl is not due to accumulation of inorganic Na⁺, K⁺ and Cl⁻. They could not find an explanation for the growth optimum at 300 mM NaCl.

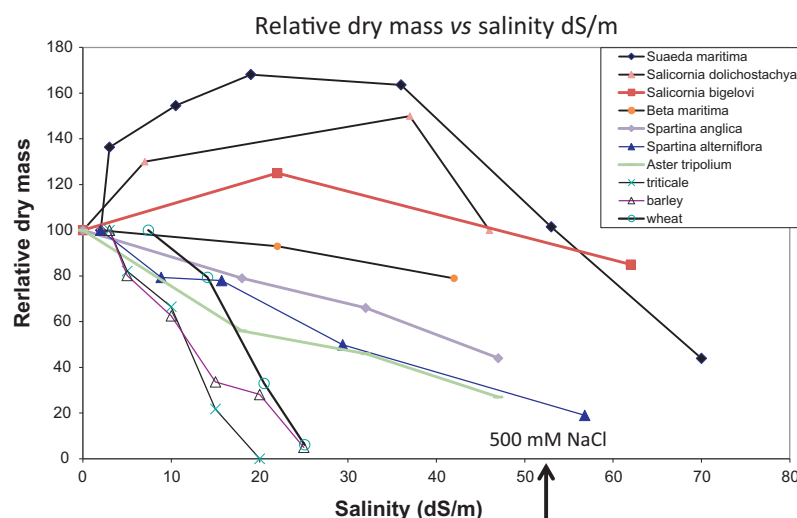


Fig. 1. Relative dry mass (dry mass at increased salinity as percentage of dry mass at 0 dS m⁻¹) in response to increasing salinity (dS m⁻¹) of salt marsh species and crops cultivated in hydroponics. Source: (1) *Suaeda maritima* (Yeo and Flowers, 1980); (2) *Salicornia dolichostachya* (van Diggelen, 1988); (3) *Salicornia bigelovi* (Glenn and O'leary, 1984; Glenn et al., 1991); (4) *Spartina alterniflora* (Parrondo et al., 1978); (5) *Spartina alterniflora* (van Diggelen, 1988); (6) *Aster tripolium* (van Diggelen, 1988); (7) *Beta vulgaris* ssp. *maritima* (Rozema et al., 1993; Niazi, 2007); (8) Triticale and Barley (*Hordeum vulgare*) (Sajjad, 1983); Wheat (*Triticum aestivum*) (Francois et al., 1994).

To our knowledge this salt stimulated growth is only found in indoor studies with precise and constant salinity levels in nutrient solutions and in sand-grown tanks systems (Wetson, 2008), but not in field studies. Therefore, salt stimulated growth may reflect an artifact of the indoor cultivation method. Perhaps because of the possible constitutive nature of the salt tolerance mechanism in these extremely salt tolerant species, a limited availability or absence of NaCl may disturb the general metabolism and nutrient homeostasis, exemplified by over-accumulation of Fe^{2+} (H. Schat, unpublished). Nutrient homeostasis may then be restored at increased salinity and the apparent salt requirement may alternatively be interpreted as impaired growth at 0 mM NaCl rather than real salt stimulated growth. At a limited availability of NaCl, Na^+ and Cl^- may be preferentially accumulated in cell walls. If so, then a low vacuolar content of Na^+ and Cl^- may lead to reduced turgor with reduced growth as a consequence. This may be restored at increased salinity allowing increased vacuolar concentrations of inorganic Na^+ and Cl^- . It may thus be misleading to speak of a salt requirement and 'obligate halophytism' (Barbour, 1970), and salt-stimulated growth. The yield-salinity response curves of *S. dolichostachya*, *S. bigelovii* (Ayala and O'leary, 1995 and Suaeda maritima (Yeo and Flowers, 1980) could also be described in terms of growth reduction in the absence of salt (Fig. 1).

If not the above 'artefacts' can be held responsible for salt stimulated growth or depressed growth at low salinity, it remains unknown as yet which part(s) of the metabolism of *Salicornia* perform better at say 300 mM NaCl than at low salinity. It appears that salt requirement for optimal enzyme activity and membrane stability can be ruled out.

4. Salt tolerance of dicotyledonae and monocotyledonae (Poaceae), high-salt tolerance and salt stimulated growth linked with succulence?

4.1. Halophytes, xerophytes, succulence and salt tolerance of dicotyledonae and monocotyledonae

To our knowledge, within the dicotyledonae there is no NaCl requirement for growth known for groups other than the halophytic Salicornioideae, Chenopodioideae and Suaedoideae (Section 3.1). It also appears that the NaCl stimulated growth in these halophytic plant groups is associated with high levels of salt tolerance of these plant groups, which are the highest among salt tolerant angiosperms (Waisel, 1972; Waisel et al., 2002; Rozema and Flowers, 2008). Does the (virtually) absence of salt stimulated growth in (non-succulent) halophytic Poaceae provide a clue to the understanding of salt requirement? Could it be that succulence is a prerequisite for salt-stimulated growth? It brings us to compare salt tolerance of dicotyledonae and monocotyledonae (Poaceae) and to consider succulence in some more detail.

The water relationships and growth strategies of Poaceae and dicotyledonae, including their halophytic representatives, differ (Rozema et al., 1987a,b; Rozema, 1991). The position of the growth meristem of new leaves in dicotyledonae is at the shoot apex and for Poaceae at the leaf and shoot base. Monocotyledonous Poaceae and dicotyledonae differ also in cell wall thickness, the modulus of elasticity, cell wall loosening and wall extensibility. Such factors may relate to the NaCl stimulated growth in halophytic Chenopods. If salt stimulated growth in terrestrial plants were to be linked with succulence in dicotyledonae, then elasticity of cell walls and associated water relationships may be involved (Rozema, 1991; Hasegawa et al., 2000). The above NaCl requirement of *Salicornia* and other halophytic chenopods at 300 mM NaCl does not occur within halophytic monocotyledonae. Perhaps with the exception

of the halophytic monocotyledonae *T. maritima* (Juncaginaceae), which is fairly succulent (Jennings, 1968) and shows growth stimulation at 50 mM NaCl. Also among the Agavaceae and Aloaceae there are a number of halophytic succulents, and seagrasses also belong to a different salt tolerant group of monocots. However, since not all succulent dicotyledonae halophytes show salt stimulated growth, e.g. *A. tripolium* (Fig. 1), succulence as such does not seem to be sufficient to explain salt stimulated growth in halophytes, although it might well be essential.

It has been argued that succulence in halophytes, also called 'electrolyte succulence', would be a component trait of the salt tolerance syndrome. However, valid evidence either against or in favor of this viewpoint is presently lacking. It has been argued that succulence represents an adaptation to 'physiological drought' caused by the low water potential of the environment. It has also been argued that halophytes might have evolved from xerophytes (cf. Waisel, 1972). Halophytes would have conserved their xerophytic traits because of the physiological drought imposed by a saline environment. However, halophytes and glycophytes appear to maintain comparable water potential gradients between their body and the environment, which implies that they should experience a comparable degree, if any, of drought. Moreover, electrolyte succulence among halophytes is not associated with the low rates of cuticular transpiration (H. Schat, unpublished) and growth typically found among xerophytes, which further casts doubt on the 'physiological drought hypothesis'. Anyway, whether electrolyte succulence contributes to salt tolerance and if so, by which mechanism, is completely unknown and probably very hard to unravel because of a general lack of intraspecific variability (see Sections 6 and 7 in this paper). Here, we conclude that physiological basis of the NaCl stimulated growth in halophytic Chenopods is still elusive; we only assume that succulence might be a prerequisite for salt stimulated growth.

4.2. Na^+ and K^+ uptake and water relationships of halophytic dicotyledonae and monocotyledonae

Halophytic Poaceae, have a much higher K^+ over Na^+ selectivity than any dicotyledonous halophytes (Flowers and Colmer, 2008; Munns and Tester, 2008). This may suggest that Poaceae halophytes would rely on improved K^+ homeostasis and/or Na^+ exclusion under salt exposure, rather than on an enhanced capacity to sequester plant-internal Na^+ ions as in halophytic dicotyledonae. However, although Na^+ exclusion is undoubtedly the most important determinant of salt tolerance variation among Poaceae, there is strong evidence that "Na⁺ tissue tolerance" can play an important subsidiary role (Colmer et al., 2006). Na^+ exclusion in salt tolerant Poaceae halophytes implies the production of organic osmotic solutes such as carbohydrate polyols to maintain a balance of internal (tissue level) and external water potentials in a saline environment. Investment of organic osmolytes in Poaceae halophytes for osmoregulation (other than the exploitation of inorganic Na^+ and Cl^- for osmoregulatory functioning in dicotyledonae) will be at the cost of biomass production. Osmolytes are also counted toward accumulated biomass. The building of the enzymatic pathways required for their synthesis, however may reduce the amount of photosynthetic and other growth related processes, and thereby reduce growth. In contrast with the apical growth meristem in dicotyledonae, the basal meristem in grasses is less exposed to the evaporative demand of the atmosphere which may contribute to an increased water use efficiency and growth being less sensitive to an environment with a large evaporative demand. Cell walls of Poaceae halophytes are more rigid than those of halophytic dicotyledonae (Bolanos and Longstreth, 1984) with a higher elasticity.

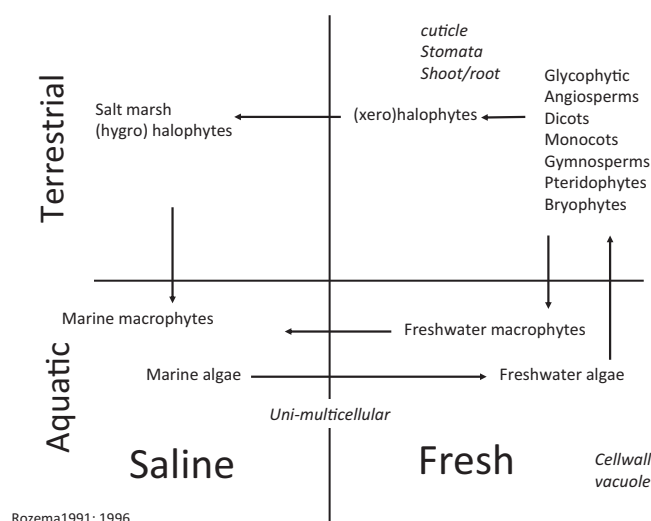


Fig. 2. Evolution of salt tolerance in terrestrial lower and higher plants, through the line marine algae → fresh water algae → 'lower' terrestrial plants → higher terrestrial plants. Hygro-halophytic salt marsh plants have secondarily evolved tolerance to seawater salinity. Marine macrophytes secondarily acquired adaptations to enable life and reproduction in seawater.

After Rozema (1991, 1996).

4.3. Evolution of salt tolerance in terrestrial dicotyledonae and monocotyledonae (Poaceae)

Above we discussed aspects of salt tolerance; we attempted to understand the high-salt tolerance and salt stimulated growth of some terrestrial plant groups and we noticed considerable differences in the salt tolerance mechanisms of dicotyledonae and monocotyledonae. Here we assess if analysis of the evolutionary history of salt tolerance in dicotyledonae families and monocotyledonae (Poaceae) provides a clue to the understanding of high-salt tolerance and salt stimulated growth in dicot *Salicornioideae*, *Chenopodioideae* and *Suaedoideae*.

The occurrence of NaCl stimulated growth within halophytic *Salicornioideae*, *Chenopodioideae* and *Suaedoideae* is unique among terrestrial angiosperms. With the evolution of land plants (Graham, 1993), the salt tolerance of their ancestral marine algae, consisting of a salt requirement for amongst other membrane stability and enzyme functioning, has been lost. There are some salt tolerant mosses (Flowers et al., 2010), ferns show no salt tolerance, so most likely salt tolerance secondarily evolved in angiosperms (Fig. 2, Rozema, 1991, 1996; Flowers et al., 2008, 2010).

Further evidence for this evolutionary pathway is provided by the absence of the Na^+ -ATPases in higher plants in line with evolution of early land plants in non-saline oligotrophic environments low in Na^+ and with only moderate Na^+ efflux (Flowers et al., 2010). However, Na^+/H^+ antiporters, transporting Na^+ out of the cytoplasm into the apoplast or the vacuole, are present in all the higher plant species investigated thus far, but conceivably expressed at higher levels both in (highly) salt tolerant monocotyledonae and dicotyledonae terrestrial plants (Benito and Rodriguez-Navarro, 2003). Na^+/H^+ antiport activity also occurs in plasma membranes of marine algae (Popova and Balnokin, 1992).

There appears to exist a continuum of salt tolerance degrees gradually changing from salt sensitive glycophytes to high-salt tolerant halophytes (Flowers et al., 2010). Along this continuum there may have been a change from inducible salt tolerance (levels, regulated by salinity changing with time (season) and space to constitutive high-salt tolerance linked with high salinity levels in space and time. Selective pressure for such high-salt tolerance may have existed at continents with prevailing arid conditions during

the palaeoclimate history. Under such hot and dry conditions soil salinity may have exceeded seawater salinity ($\approx 500 \text{ mM NaCl}$) (see Section 3.1). For such high-salt tolerant halophytes with constitutive salt tolerance, where ion transport compartmentation systems cannot be down regulated, disturbed cation homeostasis may lead to reduced growth under low salt conditions. 'Constitutive high-salt tolerance genes' are assumed to be constantly 'on' and cannot be switched off, while they need to be induced for 'inducible salt tolerance'.

Salt tolerance (of the inducible type) and of a lower degree than in halophytic *Salicornioideae*, *Chenopodioideae* and *Suaedoideae* evolved independently and repeatedly in numerous and various dicotyledonae and monocotyledonae plantgroups as discussed by Flowers et al. (2010). This underlines the uniqueness of the occurrence of NaCl stimulated growth, linked with marked succulence within dicotyledonae *Salicornioideae* *Chenopodioideae* and *Suaedoideae*. While salt tolerance in monocotyledonae evolved repeatedly and independently and although salt tolerance mechanisms in monocotyledonae and dicotyledonae differ (see a.o. Section 4.2), numerous similar salt adaptations occur in these two terrestrial plant groups. Differences in salt tolerance mechanism between monocotyledonae and dicotyledonae relate among other things to (a) accumulation and exclusion of NaCl including the use of Na^+ , K^+ and Cl^- and organic compounds in osmoregulation and their cellular compartmentation, (b) specificity of K^+ uptake, (c) water relations including succulence. Among the similar salt adaptations are: (a) compatible osmotic solutes, (b) salt glands (cf. Flowers et al., 2010).

Recent molecular phylogenetic studies have attempted to find and date the split in the angiospermae leading to monocotyledonae and dicotyledonae groups. It may be that the traditional taxonomical view that monocotyledonae evolved from dicotyledonae (Willis and McElwain, 2002) (and thus are evolutionary younger than dicotyledonae) is too simple.

Molecular genetic studies revealed that the traditional classification, based on morphological and anatomical characters, may not reflect the actual phylogenetic relationships (Stevens, 2001). Anyhow, the monocotyledonae (Poaceae) among the Angiospermae may have evolved during geological and climatic periods favoring recovery of plants from intensive herbivore grazing, about 40 Mya in the mid Tertiary (Willis and McElwain, 2002; Graham, 1993), by a basal growth meristem in grasses unlike the apical meristem in dicotyledonae (Rozema, 1991).

We conclude here that high (seawater salinity) constitutive salt tolerance and salt stimulated growth only occurs in highly succulent *Salicornioideae*, *Chenopodioideae* and *Suaedoideae* and not in 'grasses' (Poaceae). Otherwise traits of (inducible) salt tolerance evolved repeatedly and independently within monocotyledonae (Poaceae) and dicotyledonae, but never attained the high level of salt tolerance of succulent *Salicornioideae*, *Chenopodioideae* and *Suaedoideae*. The high-salt tolerance of the latter may be constitutive, while the first type of less high inducible salt tolerance appears to span a continuum from low to considerable salt tolerance (the slopes of curves of Fig. 1, 4–9, vary from steep (salt sensitive) to gentle (salt tolerant) and may be induced by soil salinity. Growth-salinity response curves (Fig. 1) of inducible salt tolerant halophytes (Fig. 1, 4–9) show maximum growth under low or zero salinity, while that of constitutive salt tolerant halophytes (Fig. 1, 1–3) demonstrates reduced growth under low or zero salinity and maximum growth at increased salinity. We realize that the distinction between inducible and constitutive salt tolerance is a provisional concept which needs to be tested. In particular, it would be interesting to see whether constitutive salt tolerance is associated with a constitutive expression of particular genes that require induction by salt exposure in inducible salt tolerant halophytes, comparable to the constitutive expression in the halophyte *Thellungiella*

halophila of particular genes that require induction by salt exposure or other stresses in *Arabidopsis thaliana* (see below).

5. Evolution of salt tolerant and salt requiring Salicornioideae

When and how did salt tolerance of terrestrial higher plants evolve? Among monocotyledonae and dicotyledonae angiosperms it did evolve repeatedly and independently (see Section 4.2). This implies that throughout the palaeo-climate and environment history of the earth selective pressure for adaptation to salinity has repeatedly occurred. Coastal salt marshes represent one such specific saline habitat with tidal flooding by seawater. Alternatively, periods in the palaeo-climate history with prevailing aridity, where hot and dry conditions occurred repeatedly and possibly with long duration, caused increased soil salinity in huge inland areas of continents. Such warm, arid climate periods with high soil salinity at times exceeding seawater salinity levels (≈ 500 mM NaCl) may have triggered evolution of high-salt tolerance in Salicornioideae, Chenopodioideae and Suaedoideae. Unfortunately, there is only indirect evidence of past soil salinity levels on continents. Past soil salinity levels under arid conditions, which may have remained high for long periods, may have led to evolution of constitutively high-salt tolerance of plants. Such constitutively high-salt tolerance requires permanent functioning of transport and compartmentation enzymes. Enzymes and processes relevant for plant growth in a low salt and oligotrophic environment may have been lost (see Section 4.2). In hydroponic studies growth of such high-salt tolerant plants under low salinity may be depressed as a result of disturbed cation homeostasis (see Section 3.1).

In the next section we focus on the evolution high-salt tolerant Salicornioideae as quite recently novel results of molecular phylogeny, palaeoclimate, diversification and biogeography research of this subfamily appeared. It could be that this phylogenetic analysis will provide better understanding of the unique high level of salt tolerance of succulent Salicornioideae.

5.1. Molecular phylogeny, palaeoclimate, diversification and biogeography

Salicornioideae and Chenopodioideae form part of the Amaranthaceae, a plant family of which numerous members occur in semi-arid, saline and sometimes disturbed regions in temperate and subtropical climate zones. Based on macro-fossil data and molecular phylogenetic research, the evolutionary roots of this group date back to circa 65 Mya (Kadereit et al., 2003) (Table 2). Kadereit and co-workers have analyzed diversification, biogeography and evolutionary trends in leaf and flower morphology of Salicornioideae and Chenopodioideae. About 90 species have been described occurring worldwide in coastal and saline habitats (Kadereit et al., 2006). Using maximum parsimony and maximum likelihood, a fossil-calibrated molecular clock and lineage through time plots, it was demonstrated that the monophyletic Salicornioideae originated in Eurasia along the northern margin of the Tethys Sea during the Late Eocene and Early Oligocene (38.2–28.7 Mya, Table 2, Kadereit et al., 2006) and experienced a rapid radiation into its major lineages during the Early Oligocene. This was after the end of warm humid period of the Middle Palaeocene to Early Eocene and the start of a long period of decreasing temperatures and increasing aridity (Willis and McElwain, 2002). High-salt tolerance of Salicornioideae might have evolved at seawater flooded coastal fringes. A high intercontinental dispersability was tracked in Salicornioideae in particular in the groups of *Salicornia* and *Sarcocornia* lineage with multiple colonization events in America, Australia and South Africa linked to

the global aridification during the Oligocene, Late Miocene and Pliocene.

5.2. Change of life form, polyploidization and inbreeding

The evolutionary history of the hygrohalophytic *Salicornia* lineages involves an evolutionary change from a tall, perennial *Sarcocornia* species, sensitive to frost and occurring in inland (non-flooded) locations, to an annual and less tall life form. The perennial salt tolerant species were geographically confined to warm temperate and Mediterranean climate zones without frost and with frequent and prolonged floodings (Kadereit et al., 2007). The geographical distribution of perennial *Sarcocornia* usually does not exceed the 1 °C January isotherm in the Northern Hemisphere.

It is likely that the frost sensitive local *Sarcocornia* lineages might have gone extinct during the Pleistocene glaciations, particularly in the Northern Hemisphere where the genus is species-poor with only two species in Eurasia and three to four species occurring in North America. In contrast, the annual *Salicornia* species have a much wider distribution in the Northern Hemisphere than *Sarcocornia*.

A *Salicornia* lineage split from *Sarcocornia* was dated 9.4–4.2 Mya, when it was globally warmer than at present, with a higher sea level as a consequence. Such annual *Salicornia* lineages occurred in Southeast Asia along the shallow coast of an inland sea at the current location of the Black Sea. It has been hypothesized that the annual life form evolved from a perennial lineage in marginal habitats with increased occurrences of frost as the driving selective force. An alternative hypothesis assumes that this evolution took place under warm climatic conditions (Kadereit et al., 2007) and that the acquired annual life form also enabled colonization of seasonally (winter) flooded areas. At the same time such adaptation to seasonal (winter) floodings also appeared to be an appropriate adaptation to habitats with severe frost.

Equipped with this annual habit, reduced size, and less sensitive to frost and flooding combined with inbreeding and polyploidization enabled extension of geographical ranges. *Salicornia* lineages are thought to have colonized cold temperate and eventually boreal and subarctic regions with seasonally flooded and dynamic habitats as a result of selective forces of severe occurrence of frost and long term periods of floodings. Two northernmost species, *Salicornia pojarkovae* occur in north-European Russia, and *S. borealis* in northern Canada along the Hudson Bay (Table 2).

Of course, this inferred change from frost sensitive perennial *Sarcocornia* lineages to an annual *Salicornia* form, not suffering from frost and seasonal and tidal floodings, requires more ecological and physiological research. It has been inferred from molecular phylogenetic analyses (Kadereit et al., 2007) that evolutionary diversification of extant *Salicornia* lineages started in the late Pliocene/early Pleistocene, about 1.8–1.4 Mya and that polyploidization played an important role. Tetraploid ($2n=36$) *Salicornia* species originated more than once from diploid ($2n=18$) *Salicornia* lineages. The success of polyploidy may lie in increased genetic redundancy supporting subsequent genetic diversification. Doubling the genome does not generate diversity per se. However, recent studies show that rapid genomic rearrangements and changes in DNA modification and gene expression patterns are associated with polyploid formation. Tetraploid ($2n=36$) hygrohalophytic *Salicornia* species used in saline agriculture are the long branched *S. dolichostachya* (occurring in the lower marsh, Huiskes et al., 1985; Rozema et al., 1987a,b; Schat et al., 1987) and *S. bigelovii*, while the short branched *S. brachystachya* (= *ramosissima*) from the upper marsh is diploid ($2n=18$). Furthermore the vast geographical distribution of annual *Salicornia* may relate to its plasticity in life-history traits, annual habit, predominant selfing, high seed

Table 2

Phylogenetic analysis of the evolution of frost tolerant, annual, hygrohalophytic *Salicornia* lineages from perennial frost sensitive *Sarcocornia* lineages, enabling colonization of colder hygrohalophytic (coastal) habitats in the Northern Hemisphere. Mya = million years ago, time estimates based on molecular clock analysis calibrated with macro fossil findings. Based on Kadereit et al. (2003, 2006, 2007).

Climate and geographical distribution	Warm temperate, Mediterranean	Cold Temperate, boreal and subarctic
Northern Hemisphere	Inland, coastline inland seas	Expansion to coastal salt marshes and colder regions
Events evolutionary processes	Perennial, northern <i>Sarcocornia</i> lineages extinct during Pleistocene glaciations Perennial to annual life form under selection pressure adaptation to frost and/or winter flooding	Diversification by polyploidization inbreeding long distance dispersal Colonization cold and hygrohalophytic habitats Northern Hemisphere
Life form	Perennial Xerophalophytic	Annual Hygrohalophytic
Species	<i>Sarcocornia perennis</i>	<i>Salicornia 'crassa'</i> <i>Salicornia borealis</i> <i>Salicornia pojarkovae</i>
Ecophysiology	Frost sensitive	Insensitive to severe frost
Time scale	38.2–28.7 Mya origin Salicornioideae (Amaranthaceae) 14.0–9.4 Mya lineage split <i>Salicornia</i> from <i>Sarcocornia</i> 9.4–4.2 Mya origin annual life form <i>Salicornia</i> from perennial life form 1.8–1.4 Mya diversification of extant <i>Salicornia</i> lineages	

set, seed polymorphism, high germination rates and good seedling establishment (Ungar, 1982, 1987).

5.3. Worldwide distribution of halophytic *Sarcocornia* and *Salicornia* species

Halophytic *Sarcocornia* and *Salicornia* have attained almost worldwide distribution through dispersal between continents. This was likely favored by the predominant occurrence in coastal habitats and their adaptation to dispersal by salt water and water birds (Wilson, 1980, 1984; Ungar, 1982, 1987). Especially *Salicornia*, which diversified only 1.8–1.4 Mya has been successful in colonizing temperate and subtropical regions almost world-wide within a relatively short time. Most other genera within the Amaranthaceae are restricted to inland salt marshes, which makes long-distance dispersal more unlikely (Kadereit et al., 2007).

Despite the relatively old age of the subfamily Salicornioideae of and its obvious adaptive superiority in extreme hygrohalophytic habitats, most genera of this subfamily are small (Kadereit et al., 2007). One explanation for this is that the sympatric occurrence of salicornioidean genera together with the low number of ecological niches in their extreme habitats, such as coastal salt marshes, hindered radiations in most taxa. Along European coastal salt marshes several *Salicornia* species, e.g. the tetraploid *S. dolichostachya* ($2n=36$) and the diploid *S. brachystachya* occur in sympatry but ecologically separated. Reproductive isolation between locally adjacent or even intermingled species may be achieved by high levels of selfing. High-salt tolerance allowed *Salicornia* species to grow in daily seawater flooded lower parts of coastal salt marshes, not accessible to other salt marsh halophytes such as *S. anglica* with a lower salt tolerance. Similarly high-salt tolerance of *Salicornia* species accounts for occurrence on saline inland sites with soil salinity temporarily exceeding seawater salinity (> 500 mM NaCl) as a result of high evapotranspiration.

5.4. Evolutionary history of hygrohalophytic *Salicornia*

In this section we analyzed the molecular phylogeny of high-salt tolerant Salicornioideae. We are intrigued by the high-salt tolerance in the plant groups of Salicornioideae, Chenopodioidae and Suaedoideae since it appears to be unique among Angiosperms. This indicates that a common ancestor of these three subfamilies acquired such high-salt tolerance. In evolutionary terms this also

means that this high-salt tolerance has not occurred repeatedly but less frequent or maybe only once.

Phylogenetic analysis taught us about variation of the palaeoclimate, diversification and biography of salt tolerant Salicornioideae. It showed a change of life form from perennial frost sensitive *Sarcocornia* to an annual life form of frost insensitive hygrohalophytic (coastal) habitats in the Northern Hemisphere. It seems likely that high-salt tolerant Salicornioideae originated in Eurasia along the northern margin of the Tethys Sea. Apparently high soil salinity under arid conditions with warm, arid climate periods with high soil salinity at times exceeding seawater levels (≈ 500 mM NaCl), acted as selective pressure for the evolution of high-salt tolerance.

This phylogenetic analysis has helped us to understand the question when and under which conditions this high-salt tolerance in these plants evolved. It has not increased the understanding of the mechanism of high-salt tolerance.

In the next section we consider the possibilities of genetic engineering of high-salt tolerance in glycophytes.

6. Breeding for salt tolerance, molecular biology and genetics of salt tolerance

Attempts to improve the salt tolerance of salt-sensitive crops through conventional breeding during the last decades have been largely unsuccessful (Flowers, 2004; Flowers and Flowers, 2005; Colmer et al., 2006; Ashraf and Akram, 2009), most probably because of the absence of sufficient genetic potential for considerable salt tolerance among the cultivars. Most of the research of Flowers aiming at increased salt tolerance of crops focuses on rice cultivars (*Oryza sativa*), that of Colmer et al. (2005) on wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), while Sabir et al. (2011) analyzed variation for salt tolerance in proso millet (*Panicum miliaceum*). These monocotyledonae crops are among moderately salt sensitive or slightly salt tolerant plants species (Fig. 1) of which the yield is about 50% reduced at a salinity of 10 dS m^{-1} , i.e. about 1/5 of the salinity of seawater. Variation of salt tolerance occurs among accessions and cultivars of such crops yet concerns low-level salt tolerance. High-level salt tolerance, however, is a complex trait, involving multiple alterations of the transport and compartmentalization of K^+ , Na^+ and Cl^- at different levels of integration, but also compatible solute synthesis, and often morphological adaptation. Therefore the genetic basis of salt tolerance can be

expected to be complex too, probably with many genes involved and, possibly, considerable epistatic interactions among them. It is unlikely that salt-sensitive crop species would accommodate the appropriate allelic variants at all of the relevant loci. Quantitative trait loci (QTL) analysis of multiple intervarietal crosses and pyramiding up QTLs may be the right way to go, but this cannot be expected to yield crop varieties with salt tolerance levels comparable with those in halophytes such as *Salicornia* (Fig. 1). In some cases, e.g. amongst Triticaceae, crop salt tolerance can be substantially improved through interspecific hybridization with related halophytic species using the protoplast fusion technique. However, the yield potential of such amphiploid hybrids appeared to be low (reviewed in Colmer et al., 2006).

7. A viable strategy for future engineering of improved salt tolerance?

7.1. Improving salt tolerance of plants: molecular genetic engineering

As an alternative for classical or marker-assisted breeding and selection or interspecific hybridization, genetic engineering is considered to be a promising strategy for salt tolerance improvement (Ashraf and Akram, 2009). However, a proper selection of candidate transgenes (Table 3) requires a detailed knowledge of the molecular mechanisms of salt tolerance in halophytes, which is presently lacking. Largely due to mutant analysis in the plant genetic model species, *A. thaliana*, a suite of genes has been identified as being essential for the wild-type salt tolerance level in this species, among which the SOS (Salt-Overly-Sensitive) genes, the Na⁺ transporter genes *NHX1* and *HKT1*, and several K⁺ transporter genes of the *HAK* family (Hasegawa et al., 2000; Flowers and Colmer, 2008; Ashraf and Akram, 2009; Shabala and Mackay, 2011). Also the genes encoding the proton-pumps of the plasmamembrane and the tonoplast are considered to be essential, because their encoded proteins are generating the electric potential and proton gradients required for passive and secondary active ion transport (Ashraf and Akram, 2009). Most of these and other genes, among which genes involved in the biosynthesis of a variety of compatible solutes or in the antioxidant machinery, as well as genes encoding transcription factors known to be involved in stress response (e.g., members of the *AP2/ERF*, *DREB*, *ZIP*, *NAC*, or *MYB* families) have been over-expressed, usually under the 35S CMV promoter, in various glycophytic hosts, including tobacco, maize, rice, wheat, potato, tomato, cabbage, and *Arabidopsis*.

7.2. Has any improved salt tolerance been reached?

Heterologous or ectopic (over-)expression of supposed 'salt tolerance genes', both of halophytic and glycophytic origin, have been claimed to improve salt tolerance in the majority of cases (e.g. Ashraf and Akram, 2009). However, most of the case studies do not allow a proper evaluation of the degree of improvement of salt tolerance in terms of an extension of the trajectory of salt concentrations that allow uninhibited growth. On the contrary, most studies used salt exposure levels that are heavily damaging for the wild-type host as well as the transformed lines (e.g. 200 or 400 mM NaCl). They merely claimed a significant quantitative difference between the degrees of growth inhibition, leaf senescence or mortality rate. This suggests nothing more than that the transformed lines experienced a lower degree of stress. Such results do not necessarily reflect an extension of the range of salt concentrations that allow normal growth and development. It is also unfortunate that most studies did not include a reference halophyte, as a positive control. Anyway, in so far as proper evaluation is possible, it appears

that the gain in salt tolerance obtained through single gene over-expression in a glycophyte background is rather inconsiderable, in comparison with the salt tolerance levels in halophytes. This is not surprising in view of the presumed complex polygenic nature of salt tolerance. It is much more surprising that single gene transformations do so often work. This could be taken as an indication that most of the individual genetic determinants of salt tolerance may have additive effects, at least in part, suggesting that pyramiding transgenes may be a viable strategy for future engineering.

7.3. Genetic breeding and mechanisms underlying salt tolerance in halophytes

To select the proper transgenes, it is indispensable to learn more about the mechanisms underlying salt tolerance in halophytes. Several investigators have used candidate gene cDNAs from halophytic species for over-expression in *A. thaliana* or other glycophyte hosts, usually under the 35S CMV promoter, and concluded, in case of significant alleviation of salt stress in the transgenic hosts, that the genes in question do contribute to the salt tolerance of the halophytic source species. This might well be true, but as such not very relevant. The important question is whether the genes in question contribute more to salt tolerance in the halophyte than the orthologous genes do in glycophytes. In other words, whether these genes are responsible for at least some part of the difference in the degree of salt tolerance between a halophyte and the glycophyte reference.

Of course there will generally be structural differences between the proteins encoded by any pair of orthologous genes from halophytes and glycophytes, albeit only through selectively neutral substitution. Thus far, there are no indications from the literature that any structural differences between orthologous halophyte and glycophyte proteins would yield significantly different contributions to the host's salt tolerance, as long as their encoding genes are expressed under the same promoter. On the contrary, in comparisons available so far, halophyte transgene cDNAs do not appear to confer more salt tolerance than their glycophyte orthologs (e.g., Chang-Qing et al., 2008; Li et al., 2008; Li et al., 2011). It is obviously much more likely that halophytes achieved their superior tolerance through alteration of the expression patterns of particular genes (e.g. Aleman et al., 2009), rather than non-synonymous substitutions in the coding regions, in conformity with the popular, but still controversial hypothesis that micro-evolutionary change proceeds mainly via alteration of cis-regulatory sequences (Wittkopp et al., 2004; Hoekstra and Coyne, 2007). To test the applicability of this hypothesis to halophyte evolution, it is necessary to perform halophyte/glycophyte promoter swaps and to compare the activities of the corresponding promoter: GUS constructs, to check for possible differences in tissue-specificity patterns. Unfortunately, such studies have not been done thus far.

7.4. Evolution of heavy metal tolerance and metallophytes compared with salt tolerance of halophytes

It will be very interesting to compare the evolution of salt tolerance in halophytes with that of heavy metal tolerance in metallophytes, the latter having been much better studied at the molecular level.

There are several reasons to suppose that there may be similarities in the evolution of halophytes and metallophytes. First, like halophytes, metallophytes are relatively rare, and widely, though erratically and unevenly spread over genera, families and orders, which implies repeated independent evolution. Second, both halophytes and metallophytes have to cope with excessive, potentially toxic cation burdens. These have to be sequestered, at least in part

Table 3

A selection of transgenes of halophytic origin that have been claimed to enhance salt tolerance in glycophytic hosts. In all cases, the transgenes were expressed under the 35S promoter, and their contribution to salt tolerance in the halophytic source species is unknown.

Transgene	Function	Source	Host	Reference
<i>AhProT1</i>	Proline transporter	<i>Atriplex hortensis</i>	<i>Arabidopsis thaliana</i>	Shen et al. (2002)
<i>SIBADH</i>	Betaine aldehyde dehydrogenase	<i>Suaeda liaotungensis</i>	<i>Nicotiana tabacum</i>	Li et al. (2003a)
<i>SICMO</i>	Choline monooxygenase	<i>Suaeda liaotungensis</i>	<i>Nicotiana tabacum</i>	Li et al. (2003b)
<i>AhDREB1</i>	Transcriptional regulator	<i>Atriplex hortensis</i>	<i>Nicotiana tabacum</i>	Shen et al. (2003)
<i>SsPP</i>	Vacuolar proton pumping pyrophosphatase	<i>Suaeda salsa</i>	<i>Arabidopsis thaliana</i>	Guo et al. (2006)
<i>ThCYP1</i>	Stress signaling	<i>Thellungiella halophila</i>	<i>Nicotiana tabacum</i>	Chen et al. (2007)
<i>TsVP</i>	Vacuolar proton pumping pyrophosphatase	<i>Thellungiella halophila</i>	<i>Gossypium hirsutum</i>	Lv et al. (2008)
<i>AINHX</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Aeluropus litoralis</i>	<i>Nicotiana tabacum</i>	Zhang et al. (2008)
<i>ALSAP</i>	Transcriptional regulator	<i>Aeluropus litoralis</i>	<i>Nicotiana tabacum</i>	Ben Saad et al. (2010)
<i>AmDHAR</i>	Monodehydroascorbate reductase	<i>Avicennia maritima</i>	<i>Nicotiana tabacum</i>	Kavitha et al. (2010a,b)
<i>SeCMO</i>	Choline monooxygenase	<i>Salicornia europaea</i>	<i>Nicotiana tabacum</i>	Wu et al. (2010)
<i>ThZFL</i>	Transcriptional regulator	<i>Tamarix hispida</i>	<i>Nicotiana tabacum</i>	An et al. (2011)
<i>HcNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Halostachys caspica</i>	<i>Arabidopsis thaliana</i>	Guan et al. (2011)
<i>SbGSTU</i>	Tau class glutathione transferase	<i>Salicornia brachyata</i>	<i>Nicotiana tabacum</i>	Jha et al. (2011a)
<i>SbNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Salicornia brachyata</i>	<i>Nicotiana tabacum</i>	Jha et al. (2011b)
<i>SaNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Spartina anglica</i>	<i>Oryza sativa</i>	Lan et al. (2011)
<i>ScVP</i>	Vacuolar proton pumping pyrophosphatase	<i>Suaeda corniculata</i>	<i>Arabidopsis thaliana</i>	Liu et al. (2011)

through alteration of subcellular compartmentalization patterns, which are likely to be achieved through altered transmembrane transport capacities. On the other hand, sodium and heavy metal cations are transported by different transporter protein families. In so far salt tolerance would be comparable with heavy metal tolerance indeed, high-salt tolerance might be achieved mainly through copy number expansion and/or a deregulated, constitutive over-expression or repression of particular genes, usually a subset of those that are also involved in cation homeostasis or osmotic adaptation in glycophytes, rather than through changes of protein structure (Hanikenne and Nouet, 2011). There are several reports in line with this view (Taji et al., 2004; Inan et al., 2004; Kant et al., 2006; Zhang et al., 2008; Edelist et al., 2009; Sahu and Shaw, 2009).

Many studies have checked the inducibility by salt exposure of supposed candidate tolerance genes in halophytes, or made larger-scale gene expression comparisons between salt-treated and untreated halophytes (Table 4), and concluded, in case of significant induction, that the genes in question are likely to be involved in the salt tolerance mechanism of the halophyte under study (Table 4). It is unfortunate that such studies usually did not include a glycophytic species as a negative reference. Admittedly, nowadays there are a lot of data concerning gene expression in response to salinity in glycophytes, but glycophytes and halophytes have been seldomly compared in a single experiment under identical conditions, which often hampers fair comparisons. It can therefore not be excluded that genes that are salt-inducible in halophytes may equally well be salt-inducible in glycophytes. In case of 'stress-responsive' genes, it might often appear that the threshold exposure levels for induction are even higher in halophytes than in glycophytes, simply because halophytes may not yet perceive stress at salinity levels that are already stressful for glycophytes. In general, euhalophytes are more or less continuously exposed to extraordinary salinity levels and, again in analogy with metallophytes, this would be expected to lead to a more or less constitutively and strongly enhanced or repressed expression of particular genes. These genes may be expected to represent a subset of those that are also involved in the ion homeostasis or osmotic adaptation in glycophytes, in combination with a shift toward higher levels of the threshold salt concentrations for the induction of the majority of stress-responsive genes. This pattern has been observed in the salt tolerant plant genetic model, *T. halophila* (Taji et al., 2004; Kant et al., 2006). However, since *T. halophila* is a 'multiple stress tolerator', it remains unclear whether this pattern is specifically related to its salt tolerance (see below).

7.5. Improving salt tolerance of plants: identification of 'real' salt tolerance genes

As a first step in the identification of 'real' salt tolerance genes, i.e. the genes that produce the difference in salt tolerance between halophytes and glycophytes, extensive transcriptomic comparisons are therefore urgently needed. Unfortunately, such comparisons are overall lacking, except for the case of *A. thaliana* and the related halophyte *T. halophila* (currently *T. salsuginea*). These species share a high degree of DNA sequence identity, which allows a more or less reliable use of Arabidopsis-based full genome micro-arrays (Inan et al., 2004). However, although *T. halophila* is able to survive sea water salt concentrations, in terms of its threshold exposure level for growth inhibition it is certainly not among the most salt tolerant halophytes (Colmer and Flowers, 2008). Moreover, unlike specifically salt-adapted halophytes, *T. halophila* is tolerant for multiple stressors and it has the low growth rate typical of a "stress tolerator" (Inan et al., 2004). From this perspective, *T. halophila* may appear not to be the ideal model for salt tolerance gene identification.

In general, cross-species transcriptome comparisons using heterologous hybridization platforms suffer from various drawbacks owing to sequence differences (Hammond et al., 2006). These problems can nowadays be avoided using the 'deep sequencing' technique, ideally in combination with full genome gDNA sequencing. However, even intraspecific full transcriptome or proteome comparisons between plants from different populations tend to yield thousands of differentially expressed genes or proteins, the great majority of them probably without any bearing on the trait of interest (e.g., Van de Mortel et al., 2006; Tuomainen et al., 2010). Therefore, it would be desirable to be able to compare near isogenic lines with maximally contrasting salt tolerance levels, selected from a cross between a glycophyte with a conspecific halophyte. However, the opportunity to make such comparisons is probably not there, because facultative halophytes, with both halophytic and glycophytic populations, are rare. Moreover, within facultative halophytes, e.g. *F. rubra* (Rozema et al., 1978) the distinction between halophytes and glycophytes is usually set at subspecies level, and possibly associated with differences in chromosome numbers, or large-scale internal chromosomal rearrangements (Huff and Palazzo, 1998). This could seriously restrict the possibilities for effective recombination. For the same reasons, i.e. the difficulties to obtain broadly segregating crosses and efficient recombination, (candidate) gene identification through QTL analysis and fine mapping in halophyte × glycophyte crosses is probably equally problematic. In spite of these limitations, it would be very

Table 4
A selection of genes shown to be upregulated by salt treatment in halophytes and supposed to be involved in their salt tolerance mechanism. In all cases their status as a 'real' salt tolerance gene, that is, contributing to the difference in salt tolerance between halophytes and glycophytes, remain unvalidated.

Gene	Function	Species	Reference
<i>McSKD1</i>	Potassium transporter	<i>Mesembryanthemum cristallinum</i>	Yen et al. (2000)
<i>McUBC</i>	Ubiquitin conjugating enzyme	<i>Mesembryanthemum cristallinum</i>	Yen et al. (2000)
<i>McHKT1</i>	Plasmamembrane-located Na ⁺ (K ⁺)/H ⁺ symporter	<i>Mesembryanthemum cristallinum</i>	Su et al. (2003)
<i>SsNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Suaeda salsa</i>	Ma et al. (2004)
<i>AmNAC1</i>	Transcriptional regulator	<i>Avicennia maritima</i>	Ganesan et al. (2008)
<i>SsHKT1</i>	Plasma membrane-located Na ⁺ (K ⁺)/H ⁺ symporter	<i>Suaeda salsa</i>	Shao et al. (2008)
<i>FrPIP2;1</i>	Aquaporin	<i>Festuca rubra</i> ssp. <i>littoralis</i>	Diedhiou et al. (2008)
<i>FrVHA-B</i>	Vacuolar H ⁺ ATPase	<i>Festuca rubra</i> ssp. <i>littoralis</i>	Diedhiou et al. (2008)
<i>FrNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Festuca rubra</i> ssp. <i>littoralis</i>	Diedhiou et al. (2008)
<i>SpFBA</i>	Fructose-1,6-biphosphate aldolase	<i>Sesuvium portulacastrum</i>	Fan et al. (2009)
<i>SmPEM</i>	Phosphoethanolamine-N-methyltransferase, involved in glycinebetaine synthesis	<i>Suaeda maritima</i>	Sahu and Shaw (2009)
<i>McSOS1</i>	Plasma membrane-located Na ⁺ /H ⁺ antiporter	<i>Mesembryanthemum cristallinum</i>	Cosentino et al. (2010)
<i>McNHX1</i>	Vacuolar Na ⁺ (K ⁺)/H ⁺ antiporter	<i>Mesembryanthemum cristallinum</i>	Cosentino et al. (2010)
<i>McNhaD</i>	Chloroplast envelope-located Na ⁺ /H ⁺ transporter	<i>Mesembryanthemum cristallinum</i>	Cosentino et al. (2010)
<i>AmMDAR</i>	Monodehydroascorbate reductase	<i>Avicennia maritima</i>	Kavitha et al. (2010a,b)
<i>AmHA1</i>	Plasma membrane-located H ⁺ ATPase	<i>Avicennia maritima</i>	Chen et al. (2010)
<i>AmSOS1</i>	Plasma membrane-located Na ⁺ /H ⁺ antiporter	<i>Avicennia maritima</i>	Chen et al. (2010)
<i>AmNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Avicennia maritima</i>	Chen et al. (2010)
<i>AhVP</i>	Vacuolar H ⁺ pumping pyrophosphatase	<i>Atriplex halimus</i>	Khedr et al. (2011a,b)
<i>AhHA</i>	Plasma membrane-located H ⁺ ATPase	<i>Atriplex halimus</i>	Khedr et al. (2011a,b)
<i>TsP5CS1</i>	Δ1-pyrroline-5-carboxylate synthetase, involved in proline synthesis	<i>Thellungiella salsuginea</i>	Radyukina et al. (2011)
<i>CqSOS1</i>	Plasma membrane-located Na ⁺ /H ⁺ antiporter	<i>Chenopodium quinoa</i>	Ruiz-Carrasco et al. (2011)
<i>CqNHX1</i>	Vacuolar Na ⁺ /H ⁺ antiporter	<i>Chenopodium quinoa</i>	Ruiz-Carrasco et al. (2011)

helpful to compare the transcriptomes of halophytes and, so far possible, taxonomically related glycophytes, which is presently possible through deep sequencing. However, deep sequencing is still an expensive technique, which is not always affordable.

Fortunately, there is probably still a lot to discover using a simple common-sense based candidate gene approach. As outlined above, it is likely that the genes that are responsible for the superior salt tolerance in halophytes are a subset of the genes that are responsible for the monovalent ion homeostasis and the accumulation of potential compatible solutes in glycophytes.

7.6. Improving salt tolerance of plants: comparisons between the expression and regulation patterns in halophytes and glycophytes

As yet there are only a few comparisons between the expression and regulation patterns of candidate salt tolerance genes in halophytes and glycophytes. It is evident that such comparisons can provide useful clues with regard to nature of the salt tolerance mechanism in halophytes, such as shown in case studies on *SOS1* and *HAK5* in *T. halophila*, using *A. thaliana* as a reference (Kant et al., 2006; Aleman et al., 2009), and various cation transporter genes in *Helianthus paradoxus*, using *H. annuus* and *H. petiolaris* as glycophytic references (Edelist et al., 2009). The absence of more such comparative studies is undoubtedly a major omission in the salt tolerance research of the last decade. It should not be too difficult to clone the orthologs of candidate genes from halophytes, and to check their expression patterns, in comparison with a glycophyte reference species. It should also be relatively easy to clone the halophyte promoters, and to express the genes, both under the natural halophyte promoters and the orthologous glycophyte promoters, in the glycophyte mutant background. In cases where expression under the halophyte promoter provides over-complementation, or at least more salt tolerance than it does under the native glycophyte promoter, there would be a strong case for involvement of the gene in the salt tolerance mechanism of the halophyte. Of course, any other result would not mean that the gene is not involved, but merely that the higher expression in the halophyte is not due to alteration of the *cis* regulatory elements of the gene. Obviously, such experiments will never provide the undisputable hard evidence, because it is always possible to maintain that the effect of the gene might depend on the genetic background of the host. To

deliver the hard evidence, it is essential to silence the gene in the halophyte, preferably to the level at which it is expressed in the glycophyte reference. If that would lead to a loss of salt tolerance, then one could safely conclude that it is a salt tolerance gene indeed, such as demonstrated for, exclusively, *SOS1* in *T. halophila* (Oh et al., 2007).

Unfortunately, whereas a great variety of glycophyte species is available as a host for halophyte genes, *T. halophila* is the only genetically accessible halophyte thus far. However, *T. halophila* is not an ideal salt tolerance model (see above) and, in view of the probable variation in the nature and the tolerance potentials of the mechanisms in halophytes (see below), it is desirable, if not indispensable to have a number of genetically accessible halophyte model species from different monocotyledonae and dicotyledonae families. The virtual absence of genetically accessible halophytes may be owing to a high resistance to genetic transformation among frequently investigated halophyte species, but it is more likely that investigators just did not try hard enough to develop suitable transformation protocols. Effective transformation protocols are urgently required for at least a number of euhalophyte models.

As outlined before, it is likely that the salt tolerance mechanisms among halophytes may differ in various respects, both quantitatively and qualitatively. Therefore it is possible, though not necessary, that the possibilities to engineer salt tolerance in glycophytes may depend on the phylogenetic background, e.g., the family, the order, or the subclass, to which the host species belongs. For example, in view of their high K⁺ over Na⁺ selectivity, it could be predicted that over-expressing vacuolar Na⁺ pumps, like *NHX1*, should be barely effective in Poaceae hosts. However, *NHX1* of various sources has been over-expressed in a variety of Poaceae and dicotyledonous hosts, apparently with comparable effects on salt tolerance (Ashraf and Akram, 2009). This suggests that enhancing Na⁺ detoxification through vacuolar compartmentalization may be an effective strategy in both backgrounds, regardless of the fact that naturally selected salt tolerance in Poaceae halophytes is undoubtedly mainly based on improved Na⁺ exclusion (Colmer et al., 2006). In so far any as phylogenetically biased morphological mechanisms would be involved, e.g. succulence or cell wall elasticity, it will probably be very difficult to engineer them, because their genetic basis is, as yet, completely unknown, and most probably too complicated.

8. Perspective of saline agriculture and salt tolerance engineering

8.1. The need to obtain highly salt tolerant crops

Saline agriculture implies the application and irrigation of brackish and saline water for the cultivation of crops. Salinity of the irrigation water may vary from low up to that of seawater. Glasswort, *Salicornia* spp. has become a favorable, attractive and tasteful saline vegetable crop year-round available in supermarkets. Such *Salicornia* vegetable crop may be irrigated with brackish water and seawater and demonstrates a high-salt tolerance (Fig. 1). Remarkably the RGR of this halophytic crop even at low salinity is similar to that of glycophytic species and crops. Based on this trait productivity of seawater irrigated *Salicornia* could be similar to that of crops in freshwater grown conventional crops.

Similarly the salt marsh halophyte Sea aster, *A. tripolium* is and has been cultivated. For this type of saline agriculture, salt tolerant native halophytic species are being used, domestication may be needed among other things to enable mechanical sowing and harvesting. Commercially, cutting of *Salicornia* shoots during the summer growing season at coastal European marshes remains attractive (cf. De Vos et al., 2012; Ventura and Sagi, 2012). In Pakistan salt tolerant fodder beet is being grown as a cattle fodder crop in the winter period. Salt tolerance of the domesticated fodder beet and the native coastal halophyte sea beet appeared not to differ (Rozema et al., 1993; Niazi, 2007). *Chenopodium quinoa* also appears to be a promising halophyte crop (Koyro and Eisa, 2008; Jacobsen, 2003; Adolf et al., 2012). The use of salt tolerant native halophytes for saline agriculture still remains a realistic and fruitful approach whether or not after domestication.

8.2. Engineering of salt tolerance in glycophytic crops

As outlined above, there should be possibilities for the future engineering of salt tolerance in glycophytic crops (Ruan et al., 2010). To this end it is important, as a first step, to understand better halophyte physiology, and to identify the key processes and genes that are responsible for the difference in salt tolerance between halophytes and glycophytes. Candidate genes should be confirmed through their silencing and overexpression in halophytic and glycophytic genetic backgrounds, respectively, and validated salt tolerance genes should be pyramided in glycophytic hosts. However, the success of any salt tolerance engineering is likely to depend on the choice for suitable gene promoters. With few exceptions (Garg et al., 2002), candidate salt tolerance transgenes have always been expressed under the 35S CMV promoter, which is expected to yield a high level of expression in virtually all of the organs and tissues of the host plant.

It is obvious that the physiological functions of genes will be very often critically dependent on the correct localization of their products at the levels of organs, tissues and cells, particularly in case of transporter genes. Therefore, it is also likely that most of the results obtained with overexpression of candidate salt tolerance genes are in fact artifactual, in the sense that the organ-, tissue-, or cell-specificity patterns of expression of such 35S-regulated transgenes will be very different from the natural ones. As outlined above, this may strongly interfere with the functioning of the gene products and, therefore, with the salt tolerance phenotype of the host plant. This is most clearly exemplified by the study of Moller et al. (2009), who over-expressed HKT1 in *Arabidopsis* specifically in the stele, using an enhancer trap expression system, or non-specifically under the CMV 35S promoter. It appeared that over-expression in the stele, which is the natural localization of HKT1, strongly reduced Na⁺ accumulation in the shoot and enhanced salt tolerance, whereas non-specific over-expression under the CMV 35S

promoter enhanced Na⁺ accumulation in the shoot and decreased salt tolerance. Since alterations of the *cis* regulatory sequences of particular genes may be expected to play a major role in the evolution of salt tolerance (see above), it is worthwhile to express candidate salt tolerance transgenes under natural halophyte promoters, rather than, exclusively, under the CMV 35S promoter.

8.3. Perspective of saline agriculture

Improving salt tolerance of crops such as rice, wheat, tomato and potatoes to an EC level of 25 dS m⁻¹ (brackish) or higher (50 dS m⁻¹ seawater salinity, see Fig. 1) has as yet not been realized by molecular salt tolerance engineering, as has been discussed in Sections 6 and 7. Yet, the need to obtain such highly salt tolerant crops remains in a world with a rapidly growing population and a decreasing availability of fresh water for agriculture (Rozema and Flowers, 2008). Market development for products of saline agriculture has only been started and is still limited as compared with 'conventional agriculture'.

Market development of products of saline agriculture requires large scale and efficient approaches involving among other things mechanical sowing, cultivation harvesting and processing. Such goals can be reached both by the domestication of native halophytes and through genetic engineering of salt tolerance in glycophytic crops. The advantage of the latter is that modern and low cost agricultural practices have already been reached. Realistically, successful progress of both approaches will require considerable investment of time and research funding.

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